Molecular mechanisms regulating protein kinase Cζ turnover and cellular transformation

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The regulation of protein kinase C (PKC)ζ in relation to its turnover, cell growth and transformation was investigated in Rat2 fibroblasts by over-expressing wild-type or mutant forms of PKCζ. Deletion of the pseudosubstrate site (PSS) produced the most active mutant (PKCζ Δ PSS), but mutants designed to mimic phosphorylated PKCζ had lower specific activities in an *in vitro* assay. The mutant lacking phosphorylation at the Thr-560 site (T560A) had similar specific activity to wild-type PKCζ. The T560A mutant also protected PKCζ against proteolysis, whereas phosphorylation at Thr-410 targeted it towards proteosomal degradation. Blocking proteosomal degradation with lactacystin caused the accumulation of full-length PKCζ Δ PSS, T410E, PKCζ Δ PSS T410/560E, PKCζ and T560A. Expressed PKCζ activity was paralleled by extracellular-regulated protein kinase activation, increased cell division, serum-independent growth and focus formation. These foci were seen for cells expressing higher PKCζ activity (PKCζ Δ PSS, PKCζ Δ PSS T410/560E and T560A mutants), but these fibroblasts did not show significant anchorage-independent growth. This work provides novel information concerning the role of the PSS and phosphorylation sites in regulating the activity and turnover of an atypical PKC and shows how this activity can induce cell transformation with respect to focus formation.

Key words: cell growth, cell transformation, protein phosphorylation, protein kinase C (PKC), proteosome.

INTRODUCTION

Knowledge about the protein kinase C (PKC) super-family has evolved greatly since its discovery [1–4] as a mediator for the action of tumour-promoting phorbol esters [5,6]. PKCs are classified according to allosteric activators: classical (c) PKC (diacylglycerol and Ca^{2+}), novel (n) PKC (diacylglycerol) and atypical (a) PKC [Ca^{2+}- and diacylglycerol-independent, but activated indirectly by PtdIns(3,4,5)P_3, and other lipids]. Lipid effectors bind to PKC regulatory domains and relieve auto-inhibition by a pseudosubstrate site (PSS) domain, allowing substrate access and phosphorylation [7,8]. PKCs themselves are also targets for phosphorylation-mediated regulatory events (for review, see [9]). There are three priming phosphorylation sites in cPKCs that occur in the C-terminal region: in the activation loop, at the autophosphorylation site and in the hydrophobic site (in the V5 region) [10–13]. Phosphorylation of the activation loop site is crucial for catalytic activity [14–16] and the two other C-terminal sites affect protein conformation and stability. The three sites are conserved in the cPKC and nPKCs. Phosphorylation of the activation loop site is mediated by phosphoinositide-dependent kinase 1 (PDK1) [17–20]. For PKCζ and PKCι, phosphorylation in the activation loop site is required for activity [19]. The hydrophobic phosphorylation site is replaced by a glutamate residue, but the putative autophosphorylation site (based on other family members) is present.

PKC phosphorylation not only regulates catalytic activity, but also conformation, cellular localization and protein turnover. For a cPKC (PKCα), phosphorylation at the hydrophobic and autophosphorylation sites stabilizes a 'closed' conformation of the protein and thus decreases its sensitivity to proteases, or temperature-dependent instability [21]. Based on structural predictions, the most C-terminal V5 region may well interact with upper and lower lobes of the kinase domain, thus suggesting how phosphorylation in either the activation loop or hydrophobic sites may influence protein conformation. Similarly, phosphorylation at the hydrophobic site (Ser-660) of PKCβII affects Ca^{2+} affinity [22], possibly by interactions of the V5 domain with the C2 region (Ca^{2+}-binding domain) in the regulatory domain. Phosphorylation of the autophosphorylation site promotes membrane association of nPKC (PKCδ) [23]. For cPKCs, dephosphorylation of the C-terminal sites initiates down-regulation and degradation [24–26]. For example, acute exposure to phorbol esters induces transport of activated PKCα from the plasma membrane to endosomes, by a caveolae-dependent trafficking mechanism [27]. Thus activation and de-activation of PKCs coupled with phosphorylation are highly controlled processes that modulate agonist-stimulated signalling pathways. It is not known how phosphorylation regulates aPKC turnover and degradation.

aPKCs play a role in cell proliferation and inhibiting apoptosis. aPKCs regulate differentiation of PC12 [28] and leukaemic [29] cells; phosphoinositide 3-kinase regulated growth [30], and also prevent UV-induced apoptosis [31]. Moreover, PKCζ induces MAPK (mitogen-activated protein kinase) activity [32,33] and cellular transformation [34], but how phosphorylation of PKCζ regulates these effects has not been examined. Other studies showed the role of PKCζ autophosphorylation in adipocytes [35]. The stimulatory effects of insulin and PtdIns(3,4,5)P_3 on activity were partially attenuated in T560A-PKCζ mutants, and GLUT-4 translocation was also compromised. Thus, as for Thr-410 phosphorylation, the Thr-560 site becomes phosphorylated on insulin stimulation. In the present study, we decided to use mutant forms of PKCζ to study the effects of phosphorylation on PKCζ activity, turnover and cell function.

Abbreviations used: DABCO, 1,4-diazadicyclo[2.2.2]octane; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated protein kinase; LAMP, lysosome-associated membrane protein 1; MBP, myelin basic protein; PDK1, phosphoinositide-dependent kinase 1; PKC, protein kinase C; aPKC, atypical PKC; cPKC, classical PKC; nPKC, novel PKC; PSS, pseudosubstrate site; T560A etc., mutation of Thr-560 to Ala etc.

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MATERIALS AND METHODS

Materials

DMEM (Dulbecco's minimum essential medium), penicillin, streptomycin, fetal bovine serum (FBS), and protein A–Sepharose were purchased from BRL Life Technologies (Burlington, ON, Canada). BSA, PMSF, leupeptin, aprotinin, Nonidet P40, Triton X-100, PtdIns(3,4,5)P_3 phospholipids and DABCO (1,4-diazadicyclo[2.2.2]octane) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mowiol was purchased from Calbiochem (La Jolla, CA, U.S.A.) and G418 from Clontech Laboratories (Palo Alto, CA, U.S.A.). Polyclonal anti-PKCζ (C-terminal antibody) and anti-ERK (extracellular-signal-regulated protein kinase) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); anti-LAMP-1 (lysosome-associated membrane protein 1) was from Transduction Labs (Mississauga, ON, Canada), and Texas-Red X secondary antibodies (anti-rabbit and anti-goat) and Texas-Red X phalloidin were from Molecular Probes (Eugene, OR, U.S.A.). Antibodies against calreticulin, anti-GFP (green fluorescent protein) and phospho-PKCζ were gifts from Dr M. Michalak (Department of Biochemistry, University of Alberta), Dr L. G. Berthiaume (Department of Cell Biology, University of Alberta) and Dr P. J. Parker (Protein Phosphorylation Laboratory, Cancer Research UK London Research Institute, London, U.K.) respectively. [γ-^{32}P]ATP, anti-rabbit IgG linked to horseradish peroxidase and enhanced chemiluminescence kit (ECL®) were obtained from Amersham Biosciences (Baie d'Urfé, PQ, Canada).

Construction of PKCζ mutants

PKCζ was subcloned into the N-terminally tagged pLEGFP-C1 vector from Clontech. A novel *Xho*I restriction site (and no amino acids were added) was engineered into the 5′ untranslated region just before the start codon of PKCζ cDNA (obtained from Dr P. J. Parker) by PCR to allow insertion of the entire coding region of PKCζ. Site-directed mutagenesis and PCR were used to create various mutants. Modified cDNAs were sequenced and inserted into *Not*I/*BamH*I sites (for T410A, T410E, PKCζ Δ PSS and K281M) or *BamH*I/*Xba*I sites (for T560A and T560E) of the wild-type GFP-tagged PKCζ construct. Deletion of the pseudosubstrate site (PKCζ Δ PSS) was carried out as described previously [33].

Cell culture and generation of stable cell lines

All cells were cultured in DMEM containing 10 % FBS and 1 mg/ml streptomycin/penicillin in a 5 % CO_2 humidified atmosphere at 37 °C. Stable cell lines were generated [36] and resistant clones were identified after 2–3 weeks by selection with 0.5 mg/ml G418. From a single transduction, over 50 individual clones were selected. Clones were pooled and propagated as average populations of cells. Cell lines used in all experiments were of a low passage number (maximum, P8).

Immunoprecipitation and assays for kinase activity and autophosphorylation *in vitro*

Cell lines were serum-starved overnight in DMEM containing 0.1 % BSA, or left with 10 % FBS, and cells were lysed in buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.2 mM PMSF, 15 μg/ml leupeptin, 100 μg/ml aprotinin and 1 % Nonidet P40. Equal amounts of protein (500 μg) were immunoprecipitated [24] with 2 μg of anti-GFP antibody (for PKC assays), or 3 μg of anti-pERK-1 antibody (for ERK assays) and 50 μl of protein A–Sepharose beads for 1–2 h at 4 °C. Complexes were washed twice with lysis buffer and once with kinase buffer (as lysis buffer, except for 0.1 % Nonidet P40) and then resuspended in 100 μl of kinase buffer. GFP-immunoprecipitate (20 μl) was incubated in the kinase assay (50 μl) with PtdIns-(3,4,5)P_3 lipid micelles at 22 °C for 20 min [37]. The reaction was stopped by adding 5 μl of 4 × sample buffer and proteins were separated by SDS/PAGE, or samples were placed onto Whatman P81 filters, washed in 30 % acetic acid (three times, for 15 min each) and then quantified by scintillation counting [37]. ERK activity was measured by phosphorylation of myelin basic protein (MBP). Polyacrylamide (12 %) gels were run and dried, and phosphorylated protein was identified by autoradiography and quantified by scintillation counting. To measure autophosphorylation of PKCζ, cell lysates were immunoprecipitated with anti-GFP antibody. Immunoprecipitates were incubated with [γ-^{32}P]ATP at 4 °C for 30 min prior to separating proteins by SDS/PAGE [38].

Western-blot analysis

Samples were separated on 10 % or 12 % (for ERK assays) SDS/PAGE gels followed by transfer onto nitrocellulose. Antisera used were diluted 1:2000 (for PKCζ C-terminal antibody), or 1:1000 (for PKCζ phospho-antibodies) in 0.1 % (v/v) Tween 20/PBS and incubated for 1 h at 22 °C. Proteins were visualized using ECL®. Immunoreactive protein was measured by scanning and image analysis using different exposures.

Immunofluorescence and cellular localization

Immunocytochemical staining was performed as described previously [39], except for the following modifications. Cells were fixed in 4 % (w/v) paraformaldehyde and permeablized with 0.2 % (v/v) Triton X-100/PBS. Coverslips were mounted in Mowiol containing 2.5 % (w/v) DABCO. GFP fluorescence was visualized directly and F-actin was stained with Texas-Red phalloidin (diluted 1:2000). Antibodies for other markers were used at the following dilutions: calreticulin marker (for the endoplasmic reticulum), 1:200 and LAMP-1 (for lysosomes), 1:200. Slides were viewed by confocal microscopy (model LSM 510; Carl Zeiss Inc., Thornwood, NY, U.S.A.) with a 63 × oil immersion objective using HeNe (543 nm) and argon (488 nm).

Cell growth, focus and soft agar assays

Cells were seeded at 2×10^5 cells/dish (35-mm-diameter, 6-well plates) in DMEM containing 10 % (or less) FBS. Cell growth was determined by counting trypsinized cells every day for 10 days (in duplicate) with a haemocytometer. Other cells were washed again with PBS before determining protein concentrations (bicinchoninic acid assay). The medium was changed every other day. For the focus-formation assay used, cells were seeded, as described above, and grown for 3 weeks before foci were visualized and counted. The medium was changed twice weekly.

Soft agar plates were prepared [40] and the cells were diluted 1:2 1 day prior to seeding in soft agar to ensure that cells were growing exponentially. Cells were seeded in the top agar at 1×10^5 cells/6 cm dish. Fresh medium was added weekly and colonies were observed every other day. Control experiments were performed with Ras-transformed fibroblasts [41] to detect anchorage-independent growth.

RESULTS

Assessment of PKCζ activity and phosphorylation

To assess the effects of different mutations on PKCζ activity, various constructs were stably transduced into Rat2 fibroblasts.

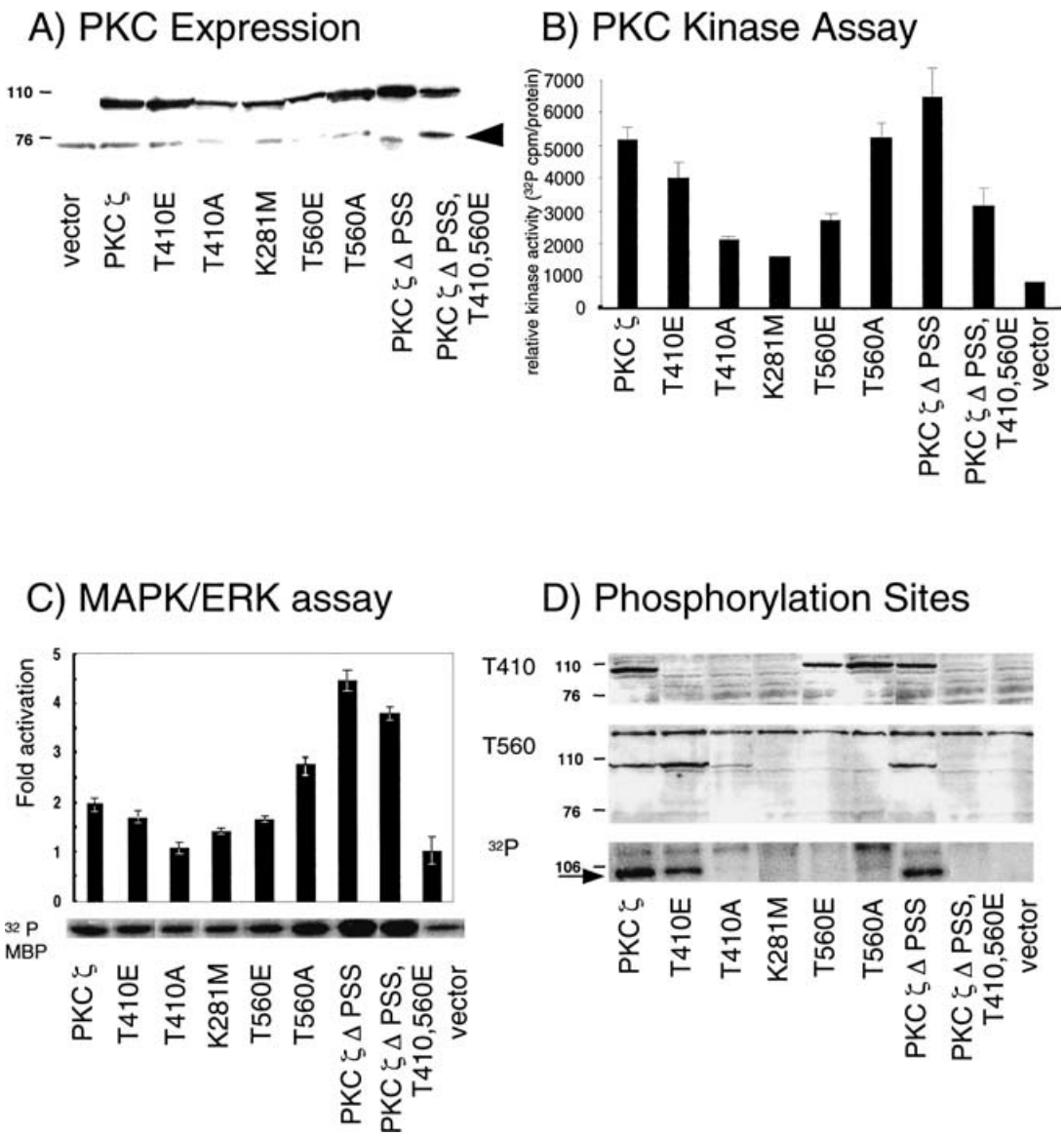

Figure 1 Characterization of Rat2 cell lines that over-express various forms of PKCζ

(**A**) Cell lysates were probed with PKCζ C-terminal antibody to detect the expression of GFP-tagged PKCζ mutants. The tag ensured easy identification and immunoprecipitation of PKCζ proteins running about 27 kDa higher than endogenous PKCζ (approx. 75 kDa, indicated by an arrowhead). (**B**) The activities of all of the mutants were estimated from immunoprecipitated protein. The activity is expressed as ^{32}P c.p.m. incorporated into MBP divided by relative PKCζ concentrations, determined by Western-blot analysis with anti-GFP antibodies of protein present in each assay (NIH image). Results are expressed as means ± S.D. for at least three independent experiments. (**C**) The activity of MAPK (ERK) was measured as a downstream target of PKCζ by determining the phosphorylation of MBP after ERK immunoprecipitation. Each precipitate was checked by Western blotting and contained approximately equal amounts of ERK protein (results not shown). Bands of MBP were cut from the gel and ^{32}P radioactivity determined, as indicated by the fold increases. Results are from a representative experiment that was repeated three times in total. (**D**) The phosphorylation status of different GFP-tagged PKCζ mutants was measured in various cell lines by, firstly, site-specific phospho-specific antibodies to the activation loop site Thr-410 (T410) and autophosphorylation site Thr-560 (T560; upper and middle panels: the phospho-antisera are not sensitive enough to detect endogenous PKCζ); and secondly, by measuring incorporation of phosphate into PKCζ in an autophosphorylation assay; the autophosphorylation sites could be detected (bottom panel). Results are representative of at least three independent experiments.

PKCζ Δ PSS is an activated form of PKCζ [33,42] and K281M is a dominant negative form [43]. We hypothesized that T410A would be inactive and that PKCζ Δ PSS T410/560E would be the most active mutant. Levels of expression for the T410A, K281M and T560E mutants were only slightly elevated, in comparison with endogenous protein. However, expression levels of the wild-type and activated proteins (PKCζ, T410E, T560A, PKCζ Δ PSS and PKCζ Δ PSS T410/T560E) were 2–5-fold higher in these cell lines (Figure 1A).

Proteins tagged with GFP were immunoprecipitated and activity was assayed *in vitro*. The specific activities of the various proteins were then normalized to the amount of each GFP-tagged PKC mutant in each assay, as determined by Western blotting of precipitated protein. PKCζ Δ PSS was the most active mutant (Figure 1B). T410E and PKCζ Δ PSS T410/560E had significantly ($P < 0.05$) lower activity than the wild-type protein. The activity profile is similar to that which we obtained in transiently transfected COS cells (results not shown), implying that stable transfection did not alter activity. Based on previous knowledge of the cPKCs [21], it could be predicted that the T560A mutant would be less active than the wild-type protein and that lack of phosphorylation at the Thr-560 site would destabilize the

protein conformation. However, the T560A mutant was as active as wild-type PKCζ and, *in vitro*, T560E was less active than wild-type PKCζ. Conversely, the T410A and K281M mutants had very low activity, demonstrating that inactive PKCζ mutants have a residual basal level of activity, like PKCδ [44]. This could be related to the lack of requirement for phosphorylation in the hydrophobic site. The PKCζ mutants also exhibited a property similar to PKCδ: the mutants were not thermally sensitive and activity was unaltered after incubation of the protein at 37 °C for 30 min prior to a kinase assay (results not shown).

Phosphorylation site mutants can be unstable on isolation from cells. Thus mutants may not be in the correct conformation to phosphorylate a substrate optimally and the assays *in vitro* may not assess PKCζ activity in the cell. Therefore, to estimate the biological activity of the mutants *in vivo*, ERK activity was measured as a downstream target of PKCζ [33]. The highest activity was seen in cell lines expressing PKCζ Δ PSS, PKCζ Δ PSS T410E/T560E and T560A (Figure 1C). Thus, the *in vitro* kinase assay (in the case of the PKCζ Δ PSS T410E/560E mutant) is not sufficiently accurate to measure the specific activity of some mutants *in vitro*. However, this measurement demonstrates a correlation between increased PKCζ activity and the ability to activate a downstream target.

Phosphorylation of PKCs in the activation-loop site by PDK1 provides a surrogate measurement of PKC kinase activity. The phosphorylation state of the various mutants was analysed by using phosphorylation site-specific antisera (Figure 1D, top and middle panels) and an autophosphorylation assay (Figure 1D, bottom panel). The antisera were specific for the phosphorylation sites and did not recognize any of the alanine or glutamate substitutions. Wild-type PKCζ from serum-stimulated cells was phosphorylated at both Thr-410 and Thr-560 sites, as was PKCζ Δ PSS. Moreover, the T560E mutant had 30 % less phosphorylation in the Thr-410 site, compared with the wild type protein. Thus, the activity of the mutants correlated with phosphorylation in the activation loop site. There are two points of note; the K281M protein was not phosphorylated in either site and the T560A protein was phosphorylated in the activation loop site, which correlates with its wild-type activity. The autophosphorylation assay showed that the T560A mutant was not autophosphorylated further. This suggests that this is the only autophosphorylation site, as was recently proposed [35]. To address how the measured activity of the mutants relates to their functions *in vivo*, the effect of different mutations on cell proliferation was examined.

Activation of PKCζ confers cellular transformation, but not anchorage-independent growth

Transformed cells exhibit faster proliferation, higher growth saturation density, reduced serum-dependency for growth, partial loss of contact inhibition for growth and anchorage-independent growth. Cells over-expressing wild type PKCζ proliferated more quickly than control cells expressing empty vector when cultured in medium containing 10 % serum (Figures 2A and 2B). Also PKCζ activation, in the form of the PKCζ Δ PSS and PKCζ Δ PSS T410/560E mutants, resulted in increased cell proliferation. This was also true for the T560A mutant. These cell lines appeared to have a higher saturation level than control cells transduced with wild-type PKCζ, or empty vector. These activated mutant cell lines also demonstrated increased growth in 0.5 % serum, compared with cells transduced with empty vector (Figure 2C). PKCζ Δ PSS, PKCζ Δ PSS T410/560E and T560A also demonstrated enhanced growth with 2 % and 5 % serum, in comparison with vector control cells and all other PKCζ cell lines (results not shown). The T560A mutant did not grow as rapidly as the PKCζ Δ PSS mutants. The morphology of these cell lines was examined. T560A, PKCζ Δ PSS and PKCζ Δ PSS T410/560E cells were smaller, more elongated, refractile and spindle-shaped, in comparison with the vector control Rat2 fibroblasts (Figure 3). Fibroblasts expressing activated PKCζ mutants exhibited properties of transformed cell lines. Cellular transformation results in multilayer formation, culminating in focus formation. After 3 weeks, such foci were seen for cells expressing T560A, PKCζ Δ PSS and PKCζ Δ PSS T410/560E (Figure 4). The foci were large and swirling in morphology. The transformed phenotype was due to over-expression of the PKCζ mutant in the foci, as determined by Western-blot analysis of foci obtained from these mutant cell lines (results not shown).

We also examined whether focus formation induced by activated PKCζ is accompanied by anchorage-independent growth. The growth of each cell line in soft agar was determined. After 3 weeks, very small colonies of cells expressing PKCζ Δ PSS T410/560E, T560A and PKCζ Δ PSS were observed. By comparison, Rat2 fibroblasts expressing Ras induced large colonies after 1 week, suggesting that fibroblasts which over-express activated PKCζ exhibit little anchorage-independent growth.

Turnover of PKCζ mutants, cellular localization and determination of the compartment of degradation

To try to explain how different mutants can cause cellular transformation, the turnover and degradation of the mutants was examined in cells by treatment with the protein synthesis inhibitor, cycloheximide (Figure 5). This treatment demonstrated the accumulation of breakdown products, one of them being the kinase domain. Wild-type PKCζ is degraded (by caspases) to give fragments of 50 kDa, 40 kDa and 25 kDa for non-tagged PKCζ [45]. After 5 h of cycloheximide treatment, for PKCζ, T410E and T560A there was little change in amounts of full-length protein (Figures 5A, 5B and 5E). Breakdown products were more abundant, suggesting their rapid turnover and degradation under normal conditions. Even after a relatively short time (2 h), distinctly lower levels of T560E and T410A were seen (40 % less). Slight decreases in PKCζ Δ PSS and PKCζ Δ PSS T410/560E full-length proteins (25 %) were also noted. After 24 h, the majority of all mutants were broken down to the kinase domain (PKCζ, T410E, T560A, PKCζ Δ PSS and PKCζ Δ PSS T410/560E) and there was no detectable protein of T410A or T560E. However, T560A had the most (30 %) remaining full-length protein. The results demonstrate that the full-length protein is normally broken down to the kinase domain fragment and that lack of phosphorylation in the Thr-560 site, combined with phosphorylation in the Thr-410 site (observed in the T560A mutant), results in a more stable full-length protein. Conversely, de-phosphorylation of the Thr-410 site decreases protein stability (T410A mutant). Phosphorylation in the Thr-560 site caused protein degradation of the T560E mutant, where there is lower Thr-410 phosphorylation (compared with wild-type PKCζ) and no dynamic Thr-560 phosphorylation. Clearly combined phosphorylation events control the life span of the protein.

To elucidate how PKCζ phosphorylation influences its function, the cellular localization of the various mutants was determined. Most mutants showed a cytoplasmic localization, with fluorescence visible on membranes including the plasma membrane (Figure 6) for active mutants (wild type PKCζ, PKCζ Δ PSS, T410E and T560A). Certain mutants (T560A, T410E, T410A and T560E) also showed distinct perinuclear localization. The identity of this region was examined using markers for

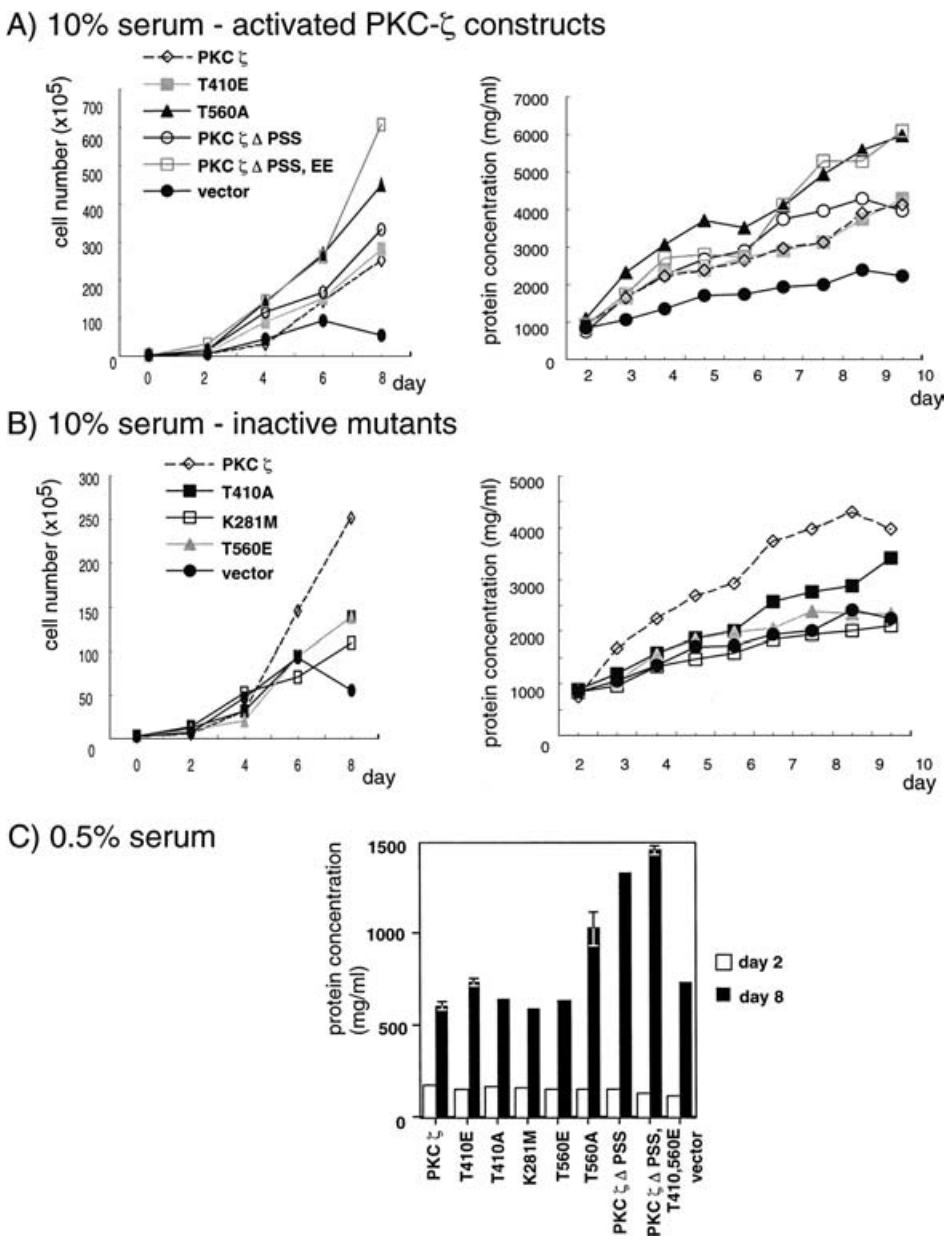

Figure 2 Growth characteristics of Rat2 fibroblasts expressing different PKCζ mutants

The growth rates of the different PKCζ cell lines were monitored over 8 or 10 days, under conditions of 10% serum (**A** and **B**), or 0.5% serum (**C**). Cells were trypsinized and counted on a haemocytometer (**A**, **B**, left-hand panels) and subsequently, protein concentration was measured (**A**, **B**, right-hand panels; and **C**). Cells grown in low serum concentrations were harvested early, after 2 days, and then after 8 days. Results are representative of at least three independent experiments. PKCζ Δ PSS, EE is PKCζ Δ PSS T410/560E.

subcellular compartments. All mutants showed co-localization with an endoplasmic reticulum marker, calreticulin (examples are shown in Figure 6A). For the sake of simplicity, results for some mutants are not shown. PKCζ and T560A are representative of the activated proteins and T560E of the inactive mutants. Inactive mutants, e.g. K281M, T410A and T560E, showed slightly less co-localization to the endoplasmic reticulum (depicted by T560E in Figure 6A). Conversely, mutants with no, or little phosphorylation in the Thr-410 site (the 'inactive' mutants) showed high co-localization with LAMP-1, a lysosomal marker (examples are shown in Figure 6B, for T410A and T560E). In comparison, the active mutants (PKCζ, T560A, PKCζ Δ PSS and T410E) showed little co-localization with LAMP-1 (the T560A mutant is used to demonstrate this in Figure 6B). A small proportion of each mutant was co-localized with giantin (a Golgi marker), and there was no obvious localization in early or late endosomes (results not shown).

The perinuclear localization of PKCζ mutants could occur for PKCζ in transit to the proteosome, since aPKCs are ubiquitinated and targeted to the proteosome for degradation [45]. To investigate this, cells were treated with a proteosome inhibitor, lactacystin (which binds certain catalytic subunits of the 20 S proteosome and inhibits the three best characterized peptidase activities of the proteosome, two irreversibly). Blocking proteosome degradation caused the accumulation of 'active' full-length proteins: T410E, T560A, PKCζ Δ PSS and PKCζ Δ PSS T410/560E (by roughly

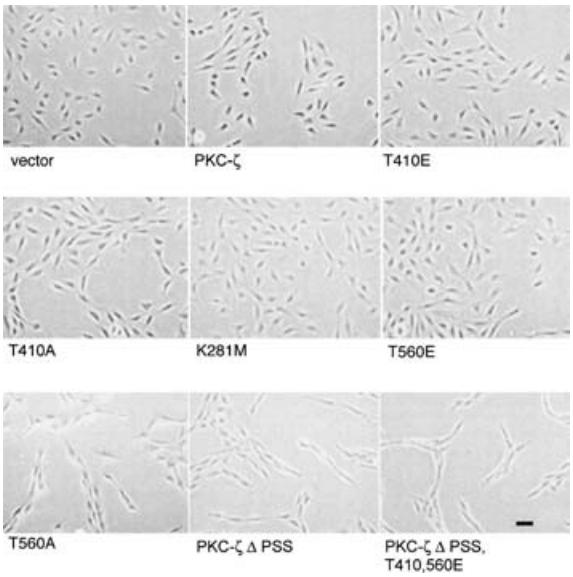

Figure 3 Morphology of Rat2 fibroblasts expressing different PKCζ mutants

Phase contrast microscopy (× 10 magnification) was used to view the morphology of the different cell lines. Representative images from three experiments are shown. Scale bar shows 20 μm.

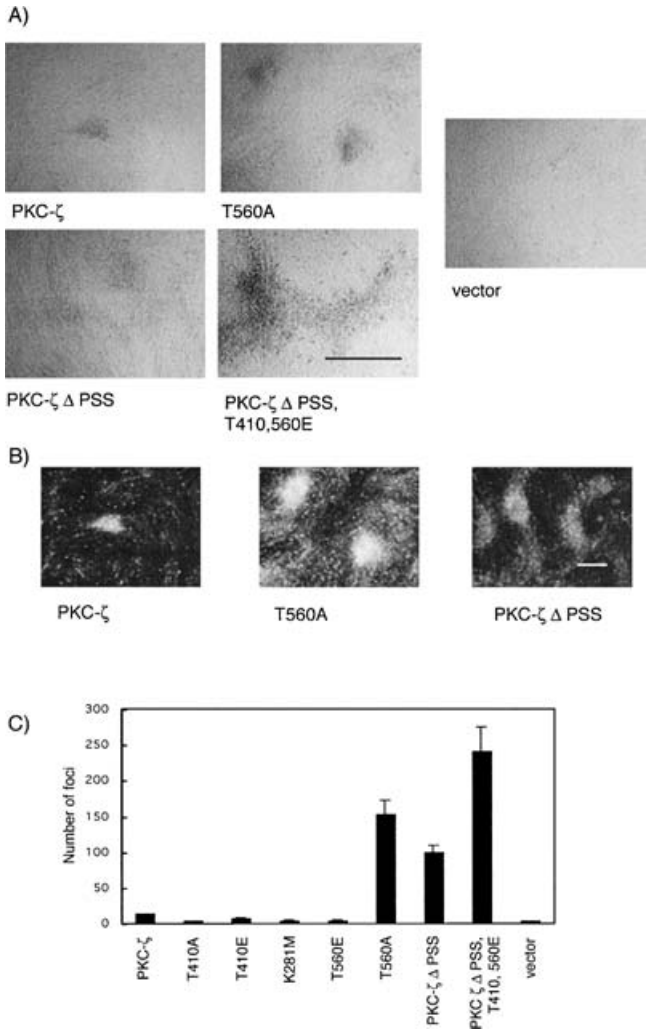

Figure 4 Focus formation

Rat2 fibroblasts expressing different PKCζ mutants were grown for 3 weeks and the formation of foci observed. Photographs were taken to demonstrate the morphology of the foci (**A** and **B**); phase contrast was used for (**B**). The numbers of foci per 6-well dish were counted for each cell line (**C**). Results are representative of at least four independent experiments. Scale bar, 3 mm.

30%) and, to a lesser extent for wild-type PKCζ (Figure 7). Cleavage products were more prevalent after lactacystin treatment, especially for T560A, where more kinase domain products were visible. The levels of proteins expressed for T410A and T560E mutants (the two mutants not, or poorly, phosphorylated in the activation loop site) did not accumulate in cells (in fact, 40% less protein was detected for T410A and 30% for T560E). This, correlates with the previous results showing a co-localization of these mutants with LAMP-1 (Figure 6B) and indicates a different degradation pathway for these mutants.

Examination of the immunofluorescence of GFP-tagged (mainly full-length) protein shows accumulation of PKCζ, T560A and to a slightly lesser extent, PKCζ Δ PSS proteins in a perinuclear localization on lactacystin treatment (Figure 8). Thus, lactacystin prevents the degradation of PKCζ mutants, which can be phosphorylated in the Thr-410 site, mirroring the results described above. For the inactive mutants (T410A and T560E), lactacystin induced a high degree of cell detachment (Figure 8G). The cells that remained attached were rounded cells and had increased peripheral actin, and often the centres of the cells were raised, presumably prior to full detachment (Figures 8E and 8F). However, little detachment and rounding occurred in cells expressing activated PKCζ (PKCζ Δ PSS, T410E or T560A), despite wild-type PKCζ showing slightly greater cortical actin than other 'active' mutants. Thus, the greater survival properties of the T560A and PKCζ Δ PSS cell lines help to explain why these cell lines showed cellular transformation.

DISCUSSION

The present study highlights novel results demonstrating how phosphorylation of aPKCs not only controls their activity, but also regulates protein turnover and degradation pathways. We showed that phosphorylation at the Thr-410 site not only confers catalytic activity, but also enhances degradation in the proteosome. Conversely, lack of phosphorylation at this site confers a preference for lysosomal degradation (as observed for the K281M and T410A mutants). As demonstrated previously, deletion of the PSS site results in a classically activated PKC, which is insensitive to other upstream signals and can activate downstream targets, such as ERK. Moreover, this results in cellular transformation. It should be noted that this may not occur *in vivo*, since *in vitro* kinase assays for PKCη Δ PSS mutants demonstrate altered substrate specificity compared with the wild-type protein, thus such mutants may be more promiscuous in substrate specificity [46]. However, for the first time, we analysed the effects of aPKC phosphorylation on cell growth. We demonstrated that lack of phosphorylation at the autophosphorylation site (Thr-560) results in an enzyme as active as the wild-type protein, but which is more stable, is less readily turned over and can confer cellular transformation. Our studies of Myc-tagged PKCζ demonstrated similar subcellular localization to that reported here (results not shown), demonstrating that the GFP tag does not adversely affect PKCζ function and distribution. Moreover, relative to the GFP-tagged PKCζ wild-type protein, different mutants exhibited different activities and transforming

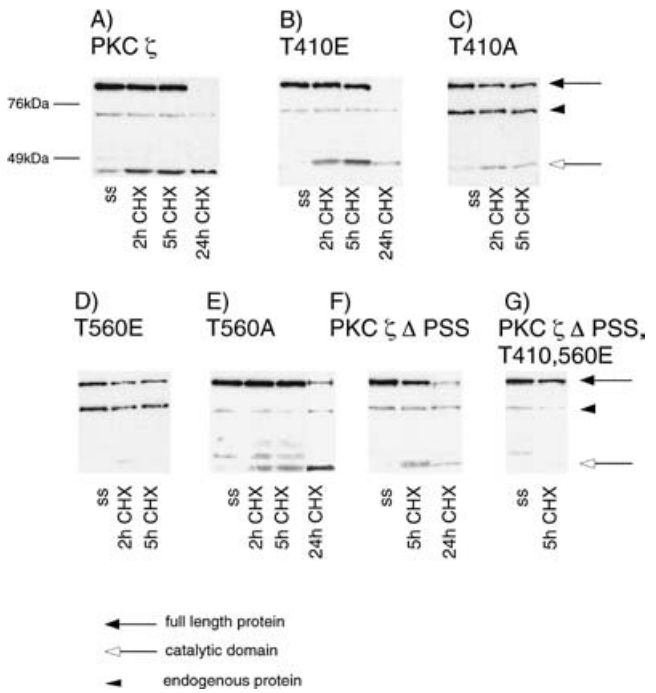

Figure 5 Stability of the PKCζ wild-type and mutant proteins

After overnight starvation (ss), various cell lines were treated with 10 μg/ml cycloheximide (CHX) for 2, 5, or 24 h. Cells were harvested in lysis buffer and protein concentrations were measured to ensure equal loading of protein per lane. Western blots were probed with the PKCζ C-terminal antibody. Full-length protein is annotated with an arrow and the kinase domain by an open arrow. A filled arrowhead denotes endogenous PKCζ. Results are representative of at least three independent experiments. The 2 h time points were not performed for panels (**F**) and (**G**).

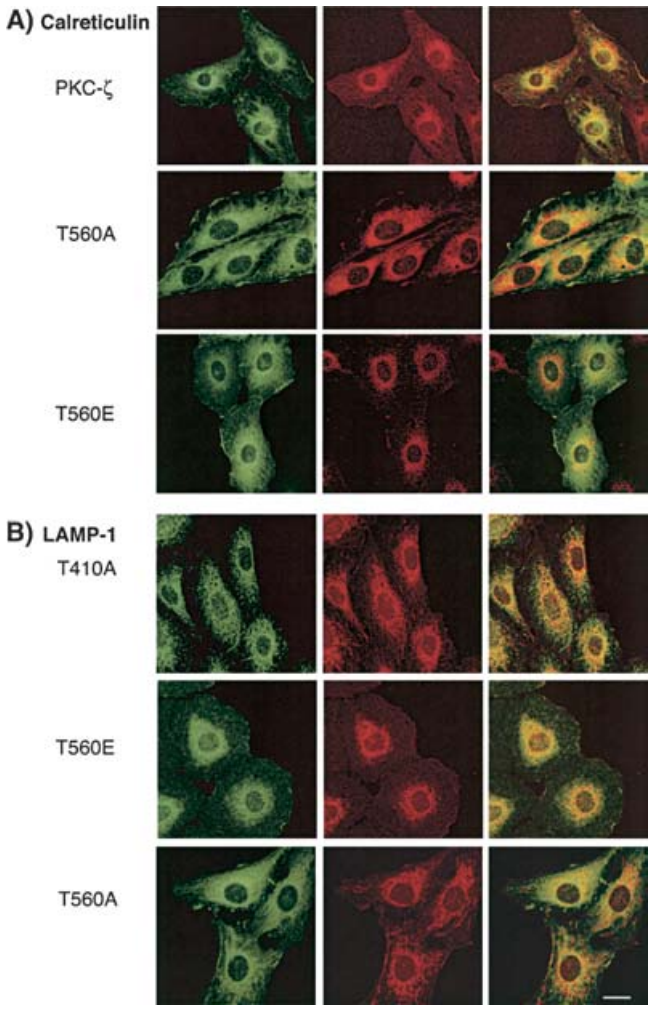

Figure 6 Cellular localization of PKCζ mutants

Cells were plated on coverslips and fixed so that the fluorescence of GFP from PKCζ mutants could be measured (**A** and **B**, left-hand column). All cell types were incubated with antibodies for the markers of the endoplasmic reticulum (**A**; calreticulin) or lysosomes (**B**; LAMP-1) and selected mutants are shown. These responses were detected with a Texas Red fluorescent secondary antibody (**A** and **B**, middle column). Merged images are illustrated in the right-hand column. All cells are shown at the same magnification and the size bar represents 10 μm. Results are representative of at least three independent experiments.

ability, which shows that the GFP tag does not influence the observed phenotypes.

Cell transformation mediated by Cdc42/Rac1 is potentiated by hPar6 [34], a binding partner for PKCζ, and this cellular transformation requires PKCζ. The authors demonstrated that complex formation (par6/Cdc42 or Rac1 with PKCζ) induces PKCζ kinase activity. This is compatible with our results demonstrating that activated forms of PKCζ (also in the form of the T560A mutant, which is less readily degraded) induce focus formation. However, this initial phase of cellular transformation by activated PKCζ by itself does not extend to anchorage-independent growth. The foci formed from T560A, PKCζ Δ PSS and PKCζ Δ PSS T410/560E cells were large and swirling, containing highly refractile spindle-shaped cells. This is similar to foci observed to occur with Raf-CAAX and oncogenic H-Ras [47]. Moreover, Qiu et al. [34] showed that Cdc42/par6/PKCζ foci were more swirling, with spindle-shaped cells, which is similar to those seen here for activated PKCζ. Thus, activated PKCζ possibly plays two different roles in transformation: upstream activation of the ERK pathway and, secondly, co-operation with Cdc42 (and par6) and downstream pathways to enable anchorage-independent growth and tumorigenesis. The second pathway must require not only an activated form of PKCζ, but binding partners to localize and sequester PKCζ correctly into an active signalling complex with Cdc42.

The fact that the T560A mutant was less readily degraded does not conform to ideas about cPKCs, where phosphorylation induces a more closed protein conformation and less accessibility to protease-dependent degradation. The aPKCs could undergo alternative regulatory mechanisms, since they do not require the first priming phosphorylation event and since they themselves can regulate nPKC and cPKC activity. For our results with the T560A mutant, we cannot rule out that compensatory phosphorylation in neighbouring sites (Thr-553 or Ser-554) confers a more stable and less readily degraded PKCζ. For PKCβII, if Thr-641 (the autophosphorylation site) is mutated, other local sites become phosphorylated to compensate, yielding a fully functional protein [48]. However, in our case, even if surrounding sites are phosphorylated to compensate in theT560A mutant, this does not explain why this mutant, and not the wild-type protein, has cellular transforming potential. However, in either scenario, dephosphorylation of the Thr-560 site, coupled with phosphorylation of the Thr-410 site by itself is crucial in regulating aPKC and its role in cellular transformation. Thus, direct (or indirectly as an initiating event) dephosphorylation of the Thr-560 site mediates activation of downstream targets and cellular transformation.

Our results also help to investigate the role phosphorylation plays in aPKC functioning. How both sites influence protein activity and conformation cannot be explained simply. The two

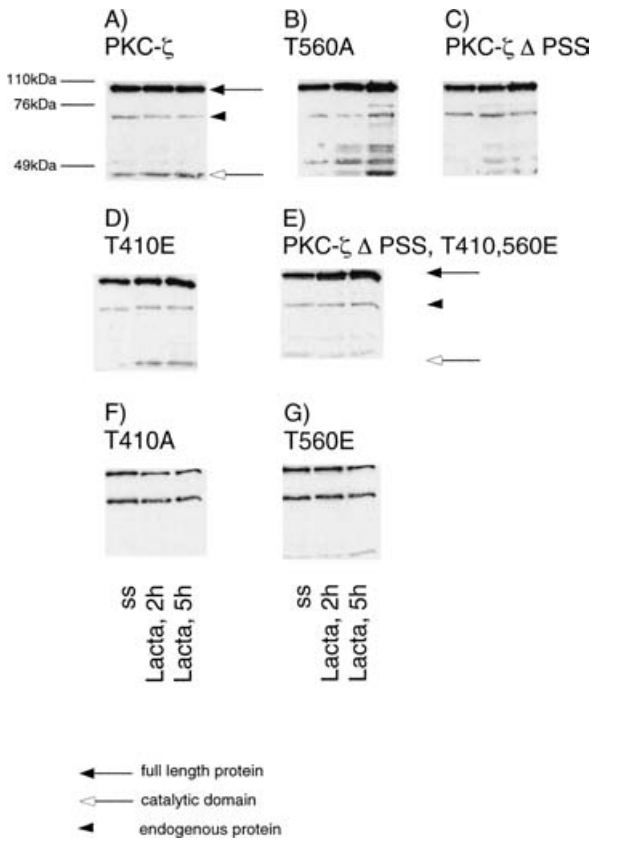

Figure 7 Degradation of PKCζ mutants

Cell lines were serum starved (ss) overnight prior to treatment for 2 h or 5 h with 10 μM lactacystin ('Lacta', a proteosome inhibitor). After harvesting in lysis buffer, protein concentrations were determined to ensure equal loading of protein for the different mutants (**A**–**G**). Western blots were probed with PKCζ antibody (a filled arrowhead denotes endogenous PKCζ). Results are representative of at least three independent experiments.

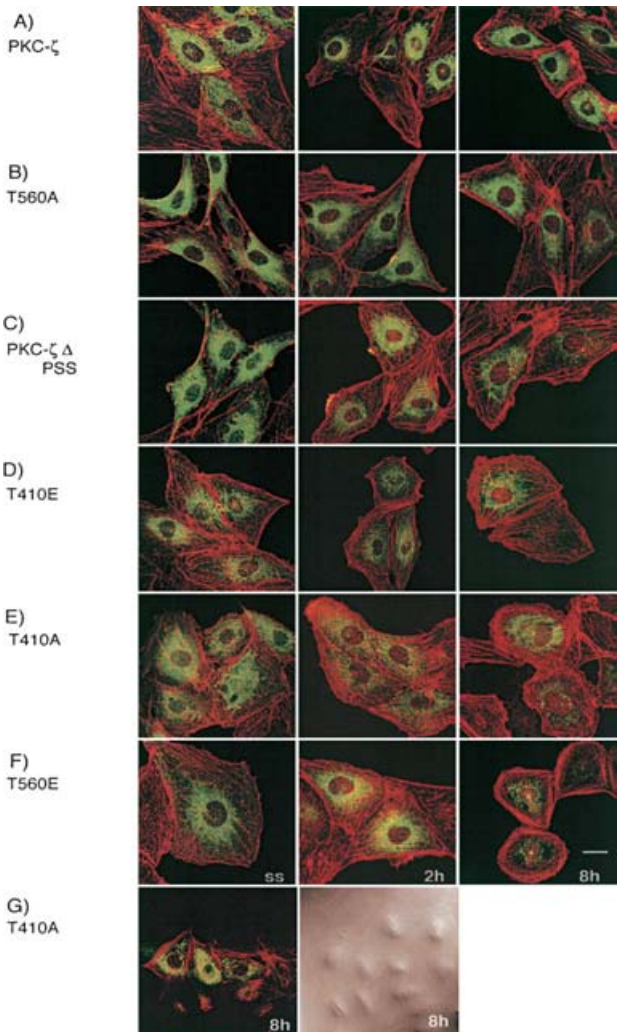

Figure 8 Lactacystin affects the cellular localization of PKCζ mutants and cell morphology

Cell lines (**A**, PKCζ; **B**, T560A; **C**, PKCζ Δ PSS; **D**, T410E; **E**, T410A; **F**, T560E) were plated on coverslips and incubated overnight in serum-free medium (ss, left-hand column) prior to treatment with lactacystin for 2 h (middle column) or 8 h (right-hand column). Cells were fixed and fluorescence from the GFP-tagged PKCζ mutants and actin filaments (stained with phalloidin) were measured. Cell detachment was observed, as shown in the phase contrast images of T410A on treatment with lactacystin (**G**, right-hand panel). All cells are shown at the same magnification and the size bar represents 10 μm. Results are representative of at least three independent experiments.

sites affect each other and understanding the dynamics of this regulation will be crucial. Phosphorylation at the Thr-410 site facilitates kinase activity; however, this event by itself is not sufficient to provide full activity. It is probable that the primary event of PKCζ activation is relief of auto-inhibition by opening up the protein conformation to reveal the phosphorylation site (Thr-410) which can thus become phosphorylated and enable full activity. Therefore, an amplitude control mechanism can be envisaged to account for the activity of various PKCζ mutants and their different cellular effects. PKCζ Δ PSS can undergo phosphorylation at the Thr-410 site, and relief of the auto-inhibition is not a transiently controlled event (as in the wild-type enzyme). It is persistent and results in constitutive activity, which may explain why this mutant has transforming potential. The lack of protein turnover of the T560A mutant and thus persistence of the protein could enable this mutant to display its transforming activity. The role that the Thr-560 site plays is not so clear cut. The T560E mutant is phosphorylated at the Thr-410 site, but has lower activity than the wild-type enzyme and is destabilized. However, the wild-type and PKCζ Δ PSS mutant were both phosphorylated at the Thr-560 site, but are more active and stable. This may indicate that transient phosphorylation controls PKCζ activity and lifespan. When this phosphorylation is not a dynamic event, the effects are more dramatic. Phosphorylation appears to control targeting to a specific degradation pathway. All PKCζ mutants that could be phosphorylated at the Thr-410 site were more prone to proteosome-mediated degradation. Lack of phosphate in this site, as associated with T410A or T560E (which has reduced levels of phosphorylation), targets PKCζ mutants to a different degradation pathway, probably in lysosomes. The PKCζ Δ PSS T410/560E 'hybrid' mutant demonstrates that the instability associated with Thr-560 phosphorylation is not overcome by deletion of the PSS, or by Thr-410 phosphorylation (as shown on cycloheximide treatment). However, indicative of the strong role of the Thr-410 phosphorylation site and deletion of the PSS, the protein undergoes proteosomal degradation and has transforming potential. Therefore, potentially, deletion of the PSS and phosphorylation of the Thr-410 site have more long-term effects on enzyme activity than Thr-560 phosphorylation. Alternatively, phosphorylation (or more importantly, dephosphorylation) of the Thr-560 site may be a more highly regulated event and enable

PKCζ to sense its external environment. Normally, this would thus prevent excessive cell growth and cellular transformation.

The autophosphorylation results implicate the Thr-560 site as being the only autophosphorylation site, since mutation of this site (T560A or T560E) did not result in any other autophosphorylation of the enzyme. A band approx. 10 kDa larger that PKCζ is seen in the Thr-560 autophosphorylation assay. This appears to be too large for a compensatory phosphorylation site, but another unknown protein could mediate T560A-induced cellular transformation. The role that the Thr-560 site plays in mediating signalling pathways could depend on the context of the protein; the upstream activating signal and subsequent downstream components. In contrast with the results presented here, T560A mutants substantially decreased insulin-stimulated haemagglutinin–GLUT4 translocation in adipocytes [35]. The identification of how dephosphorylation of this site is normally regulated in cells will present a future challenge. Previous studies suggest that the Thr-410 site, but not the Thr-560 site, is susceptible to protein phosphatase 2A (a marked increase in phosphorylation was seen in the Thr-410 site on treatment with okadaic acid, but not in the Thr-560 site; J. A. Le Good, unpublished work).

From the present results, we conclude that activation of PKCζ by either deletion of the PSS site or dephosphorylation of the Thr-560 site increases cellular proliferation, activates the ERK pathway and induces serum-independent growth and cell transformation. Our study demonstrates that protein phosphorylation has a more subtle and dynamic role than just simply regulating enzyme activity. Lack of phosphorylation at Thr-560 (combined with Thr-410 phosphorylation) protected PKCζ against proteolysis, whereas phosphorylation at Thr-410 targeted it towards proteosomal degradation. Such fine-tuning of the control of PKCζ activity may help to explain the regulation of the plethora of signalling pathways in which the aPKCs are involved.

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